ACROSOMAL SPERM PROTEIN AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to acrosomal sperm proteins for use in immunocontraception of male and female subjects and as markers for fertility.

(b) Description of Prior Art

Fertilization is a highly orchestrated process that culminates in the activation of an oocyte by a spermatozoon. Although testicular spermatozoa are fully differentiated cells, they cannot efficiently encounter the oocyte's investments. In order to acquire this property, spermatozoa must undergo post-testicular modifications within the epididymis. During this transit, the male gamete is subjected to major surface modifications such as changes in the lipids composition, acquisition of new epididymal proteins as well as post-translational modifications of sperm proteins. Taken together, these modifications are prerequisites for the spermatozoon to acquire its fertilizing ability. These processes are regulated by the epididymal luminal microenvironment, which is influenced by both epididymal and testicular protein synthesis and secretion.

Using the hamster as a model, a 26 kDa protein, the P26h, which shows immunocontraceptive properties when used to actively immunize male hamsters, has previously been described (Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, **51**: 1255-1263). This protein is localized on the sperm acrosome and is acquired during the epididymal transit. P26h plays a role in egg-sperm interactions as shown by the ability of P26h antibodies to inhibit sperm-*zona pellucida* binding *in vivo* and *in vitro* (Bérubé and Sullivan, *supra*).

It would be highly desirable to be provided with an acrosomal sperm protein for use in immunocontraception of male and female subjects.

It would also be highly desirable to be provided with an acrosomal sperm protein for use as a marker for fertility.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a use of an acrosomal sperm protein in immunocontraception of male and female subjects.

In accordance with the present invention there is provided a method of immunocontraception of a male or female subject, which comprises administering to the male or female subject an antigenic fragment of a P34 protein showing a high specificity for the P34 protein to elicit an immunocontraception response by the male or female subject, the antigenic fragment of P34.

The P34 protein used may have a sequence identified as SEQ ID NO:3 and a preferred antigenic fragment thereof includes, without limitation, MELFLAGRRVL (SEQ ID NO:4) and CHKAKTMLNRI (SEQ ID NO:5).

In accordance with the present invention, there is also provided an immunocontraceptive vaccine for a male or female subject, which comprises an antigenic fragment of a P34 protein in association with a suitable pharmaceutically acceptable carrier, wherein the vaccine elicits an immunocontraception response by the male or female subject after its administration.

In accordance with the present invention there is further provided a probe as a marker for male or female fertility, which comprises a cDNA sequence capable of hybridizing under stringent conditions with the P34 human acrosomal sperm protein.

In accordance with the present invention there is further provided a method for the diagnosis of male or female infertility, which comprises the steps of determining the amount of human P34 protein in a sperm or ovule sample, and comparing the determined amount with a fertile control sample.

The amount of human P34 may be determined using an antibody raised against the human P34 protein.

In accordance with the present invention there is further provided a kit for the diagnosis of male or female infertility, which comprises an anti-P34 antibody enzyme-labeled, an enzyme substrate and a fertile control sample.

A calibration curve for the amount of human P34 may be obtained using the fertile control sample.

For the purpose of the present invention, the expression "antigenic fragment" is intended to mean any fragment of the protein that is capable of eliciting an immune response pursuant to its administration to a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the comparison of partial amino acid sequences with the corresponding amino acid sequence of P26h with AP27 (adipsin);

Fig. 2 illustrates a Northern blot analysis of hamster total RNA from 1) testis 2) whole epididymis 3) *caput* epididymis 4) *corpus* epididymis 5) *cauda* epididymis 6) fat 7) lung 8) heart 9) liver 10) kidney 11) muscle and 12) brain, probed with a nP26h 710 bp cDNA probe (upper panel) or with a positive Cyclophilin probe (lower panel);

Figs. 3A-3C illustrate the nucleotide sequence of the P26h cDNA (SEQ ID NO:1) with its encoded protein (SEQ ID NO:2);

Figs. 4A-4B illustrates the alignment of the deduced amino acid sequence of P26h with the AP27 (adipsin) and the carbonyl reductase;

Fig. 5 illustrates an *in situ* hybridization probed with the P26h RNAs probes;

Fig. 6 illustrates the immunoprecipitation of P26h cDNA translational products;

Fig. 7 illustrates a Northern blot analysis of human total RNA from 1) testis, 2) *caput* epididymidis, 4) *corpus* epididymidis and 5) *cauda* epididymidis, probed with a P34H cDNA probe; and

Figs. 8A-8B illustrates the sequence homology of the human P34 (lower lane) counterpart of P26h (upper lane).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, the determination of the origin and of the sequencing of the encoding cDNA of the P26h sperm protein showing male contraceptive properties was undertaken. N-terminal sequencing of purified P26h and of peptides generated by partial proteolysis allowed a partial identification of the protein. The Northern blot analysis revealed that a major transcript encoding P26h was localized in the testicular mRNA and that no signal was detectable in other somatic tissues of the hamster. A hamster testis cDNA library was screened and a P26h encoding cDNA was cloned and sequenced. The P26h cDNA sequence revealed a 85% identity with the cDNA corresponding to mouse adipsin and to a carbonyl reductase. The deduced P26h amino acid sequence possesses specific domains of the Short Chain Dehydrogenase/Reductase (SDR) family proteins. Antibodies generated against synthetic peptides deduced from the cDNA sequence recognized the P26h on Western blots of detergent-

extracted hamster sperm proteins. On the other hand, *in vitro* translational products synthesized from the P26h cDNA were immunoprecipitated by a polyclonal antiserum produced against the purified hamster sperm P26h. *In situ* hybridization performed on tissues from the hamster reproductive tract revealed that the P26h was principally transcribed in the seminiferous tubules and at a lower level in the *corpus* epididymidis. P26h shows unique features of the SDR family that can be used to induce contraception in males.

Sexually mature Golden hamsters (*Mesocricetus auratus*, Charles River Inc., St. Constant, Qc, Canada) were sacrificed under CO_2 atmosphere and the epididymidis excised, defatted and dissected into *caput*, *corpus* and *cauda* segments. Tissues were frozen in liquid nitrogen and stored at -80°C until used. Testicular and somatic tissues were proceeded the same way. For *in situ* hybridization, fresh tissues were rinsed in PBS-DEPC (Phosphate buffered saline-Diethyl pyrocarbonate) and fixed at 4°C for 2h in 4% (w/v) paraformaldehyde freshly prepared in PBS. Tissues were cryoprotected by sequential incubations in 10% glycerol for 1h at 4°C under agitation and then overnight in 50% OCT. Tissues were embedded in OCT and frozen in liquid nitrogen. Cryosections of ~7µm were collected on poly-L-Lysine coated slides, air-dried at -20°C and were stored at -80°C until used.

N-chlorosuccinimide proteolysis

Proteins from *cauda* epididymal spermatozoa or from the epididymal fat pad were extracted with 0.5% Nonidet™ P40 (Sigma) and submitted to a preparative SDS-PAGE. After Coomassie blue staining, the bands corresponding to a MW of 26 kDA were excised, washed twice with H_2O and rinsed with a washing solution (50% (wt/vol) urea, 50% (vol/vol) ethanol). The polyacryla-

mide bands were incubated 30 min. in 20 mg/ml N-Chlorosuccinimide in washing solution, washed in water and incubated 3 times for 1h each in an equilibrium solution (0.0625M Tris-HCl pH 6.8, 20% (vol/vol) glycerol, 30% (vol/vol) B-mercaptoethanol, 5% (wt/vol) SDS). The bands were loaded on a discontinuous polyacrylamide gel and submitted to electrophoresis. Patterns of protein fragments were visualized by silver nitrate staining or Western blotted using a P26h antiserum (Bérubé and Sullivan, *supra*). Western blotted P26h fragments were also used for N-terminal sequencing.

Partial amino acid sequence analysis

P26h was purified and absorbed on a piece of nitrocellulose sheet. 100 µl of 50 mg/ml CNBr (Cyanogen Bromide) in 70% formic acid was added to 1 mg of the dry protein and incubated under nitrogen in the dark for 24h. Digested peptides were loaded onto a VYDAK™ reversed-phase C18 column (250 x 1 mm) which was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water and eluted with a 2-100% gradient of 0.08% (v/v) TFA in 80% acetonitrile. Fractions of 0.5 ml or smaller were collected at a flow rate of 50 µl/min. Protein sequencing was performed on aliquots from one peak by automated Edman™ degradation with a pulsed-liquid phase sequencer.

RNA extraction

Tissues were homogenized with a Polytron™ in 1.5 ml of a fresh homogenization buffer solution (4M guanidium thyocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). 1 ml of Cesium Chloride-homogenization buffer (2g of CsCl/2,5 ml) was added to the tissue lysates. This was layered on cushion solution (5.7M CsCl, 0.1M EDTA, pH 7.5) and centrifuged at 60 000g overnight. The RNA pellets were resuspended in TES solution (10mM tris-HCl, 5mM EDTA, 1%

SDS, pH 7.4) and extracted with phenol/chloroform and chloroform/alcohol isoamyl 24:1. The RNA was precipitated with 0.1 vol. of sodium acetate (3M, pH 5.2) and 2.5 vol. of ethanol 95%. The RNA pellets were resuspended in DEPC water. The RNA quality was evaluated by electrophoresis on a 1% agarose gel. All solutions were made with DEPC water.

Northern blot analysis

The total RNA (20 µg) prepared from hamster and human tissues was electrophorized on 1% agarose-formaldehyde gels and transferred to a nylon membrane (Quiagen, Santa Clarita, CA) using 20x SSC (3M NaCl, 0.3M Na-Citrate). Air dried Northern blots were UV cross-linked and prehybridized at 42°C for 4h in 50% (vol/vol) formamide, 0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005M EDTA, 2 X Denhardt's reagent [0.2% (wt/vol) Ficol 400, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) BSA], 0.2 mg/ml herring sperm DNA (Sigma Chemicals, Mississauga, ON) and 0.1% SDS. The membrane was hybridized overnight at 42°C in the same solution, to which [α-^{32}P] dCTP-labeled DNA probes were added. The membranes were then washed twice in 0.1 x SSC-0.1% SDS followed by a third wash of 30 min. at 65°C in 0.1 x SSC-0.1% SDS, and exposed on Kodak™ X-O-Mat film with intensifying screens for 6-18 h at -80°C. A RNA ladder (1.6-7.4 kb; Boehringer Mannheim, Laval, QC) was electrophorized in parallel and Cyclophilin probe was used as a constitutive internal control.

RT-PCR production of a P26h cDNA probe

The first amino acids sequence obtained (MKLNFSXLRLVTGAGKGIG) showed a high homology with the peptide sequence of adipsin, a marker of adipocytes differentiation. From the nucleic acid sequence of adipsin, two primers were selected according to OLIGO 4.01™ primer analysis software (National Biosciences,

Plymouth, MN), chemically synthesized (sense downstream 5'-GTG ACA GGG GCA GGG AAA GGG-3' (SEQ ID NO:6) and antisense upstream 5'-GCA ACT GAG CAG ACT AGG AGG-3' (SEQ ID NO:7)) and used for RT-PCR on the total RNA from hamster testis.

Briefly, 5 μg of the total testis RNA was incubated with 0.5 μg oligo deoxythymidine primer at 70°C for 10 min. in a final volume of 12 μl and kept on ice. Samples were incubated for 60 min. at 42°C in a reaction mixture containing 4 μL of 5X buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol (DDT), 1.25 mM deoxynucleotide triphosphates (dNTP) and 200 IU Super Script reverse transcriptase in a final volume of 20 μl. Expression of the P26h gene was determined by amplification of the cDNA. Each reaction contained 5 μl of RT template (or water as negative control), 1.5 mM $MgCl_2$, 1 x buffer, 0.2 mM dNTPs, 10 μM of each primer and 1.5 U Taq polymerase (Pharmacia Biotech, Baie D'Urfé, QC) in a final volume of 50 μl. The PCR cycling conditions chosen were 1 min. at 95°C, 1 min. at 60°C, 1 min. at 72°C for 30 cycles, followed by a 5 min. extension at 72°C. The reaction products were analyzed using electrophoresis on a 1% agarose gel and the bands were visualized by ethidium bromide staining.

The PCR band (~710 bp) was purified (Quiaquick; Quiagen), T-Cloned in pCR 3.5 (Invitrogen, San Diego, CA), and digested with EcoR1. The insert (710 bp) was separated from the vector and other fragments by electrophoresis on a 1% agarose gel, isolated from gel matrix with a Na45 membrane (Schleicher & Schuell, Inc.), and random-prime labeled according to the supplier's instructions using the T7 Quick-Prime™ kit (Pharmacia Biotech, Baie D'Urfé, QC) with [α-^{32}P] dCTP. Cyclophilin cDNA was also random-prime labeled using the same procedure.

Cloning and sequencing of P26h cDNA

Poly(A)$^+$RNA from hamster and human testicular tissues was purified from total RNA solution using a poly(A)$^+$RNA purification kit (Pharmacia Biotech, Baie D'Urfé, QC) according to the supplier's instructions. The cDNA library was prepared according to the supplier's instructions. Briefly, testicular poly(A)$^+$RNA was reverse-transcribed and ligated into the lambda Uni-Zap™ XR vector (Stratagene, La Jolla, CA). The lambda library was packaged and amplified using *Escherichia coli* XL1-Blue cells and screened with the random-prime labeled 710 bp P26h cDNA. The positive clones were isolated by plaque purification and the longest one (1081 bp) was subcloned into pBluescript KS+™. All nucleotide sequences were determined by the dideoxinucleotide termination method (Sanger) using T7 Sequenase v 2.0 kit. The labeled reaction products were analyzed on a DNA sequencer gel. Sequence translation was performed using Gene Jockey software (Biosoft, Cambridge, UK).

***In situ* hybridization**

Tissue cryosections were fixed with freshly prepared 4% paraformaldehyde in PBS for 5 min. at RT (room temperature), incubated for 10 min. in 95% ethanol/5% acetic acid at -20°C and rehydrated by successive baths of decreasing concentrations of ethanol diluted with DEPC-H_2O. Target RNA was unmasked by enzymatic digestion with 10 µg/ml proteinase K (Boehringer Mannheim) in PBS for 10 min. at 37°C, followed by a 5 min. incubation in 0.2% glycine. Sections were postfixed for 5 min. with 4% paraformaldehyde in PBS, acetylated with 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0, for 10 min. and finally washed with PBS.

Tissues were prehybridized for 1h with a preheated 250 µg/ml salmon sperm DNA in a hybridization

solution (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, 1x Denhardt's solution, 5% dextran sulfate, 0.02% SDS and 50% formamide). Sections were then incubated overnight at 42°C, under coverslips, with 25µl of heat-denatured antisense or sense cRNA probed with DIG (Digoxigenin: Boehringer Mannheim) according to the supplier's instructions. Coverslips were removed, the sections were washed twice in 2x SSC at RT, followed by two 10 min. washes at 42°C in 2x SSC, 1x SSC and 0.2x SSC, respectively.

Hybridization reactions were detected by immunostaining with alkaline phosphatase-conjugated anti-DIG antibobies. Nonspecific staining was blocked by incubation for 1h with 5% (v/v) heat-inactivated sheep serum in 0.2M Tris-HCl, 0.2M NaCl, and 3% Triton™ X-100. Sections were then incubated for 2h at RT with the alkaline phosphatase-conjugated anti-DIG antibodies diluted 1:1000 in blocking solution, washed with tris-HCl/NaCl buffer, and incubated with 0.1M tris-HCl, pH 9.5, 0.1M NaCl, and 0.01M $MgCl_2$. The hybridization signal was visualized after a 10-15 min. incubation with the substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (GIBCO-BRL, Gaithersburg, MD). Levamisole (2 mM; Sigma) was added to the reaction mixture to inhibit endogenous alkaline phosphatase. Slides were immersed in 1mM EDTA, 0.01 Tris-HCl, pH 7.5, washed 5 min. in H_2O, counterstained with neutral red, dehydrated through baths of ethanol, cleared in xylene and mounted with Permount (Fisher scientific, Nepean, Ontario, Canada).

Eukaryotic *in vitro* translation

In vitro translation was performed from circular plasmid DNA including the P26h cDNA. The TNT coupled reticulocyte lysate system was used according to the supplier's instructions (Promega, Madison, WI, USA).

Briefly, 0.5 μg of circular plasmid DNA was added directly to TNT rabbit reticulocyte lysate. T_3 RNA polymerase (Promega, Madison, WI, USA) and S^{35}-methionine (10 mCi/ml) were added to the translation mixture. The reaction was performed for 2h at 30°C. The *de novo* synthesized proteins were analyzed by SDS-PAGE according to Laemmli. The gel was soaked in an enhancer solution (Amersham), dried and exposed on X-Omat™ AR film (Kodak) for 6h at RT. In some experiments, the translational products were submitted to NCS proteolysis before electrophoretic analysis.

In some experiments, the translational products were immunoprecipitated using an anti-P26h antiserum. 5 μl of the translation reaction mixtures were incubated 1h at RT with the P26h antiserum (Bérubé and Sullivan, *supra*) or the control serum, both diluted in Tris-saline (50mM Tris-HCl, 150mM NaCl, pH 7.5). 50 μl of packed protein-A sepharose (Pharmacia) was added for 1h at RT. The immunoprecipitate was washed several times in tris-saline (50mM Tris-HCl, 500mM NaCl, pH 7.5) solution. The immune complexes were dissociated in SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.3, 2% SDS (w/v) and 5% (v/v) B-mercaptoethanol) and submitted to SDS-PAGE according to Laemmli. The gel was enhanced, dried and exposed on X-Omat™ AR film (Kodak) for 12h at RT.

The hamster's epididymis, like that of many mammalian species, is surrounded by a fat pad. In the mouse, an adipsin mRNA is abundant in the epididymal fat cushion. There was a possibility that P26h N-terminal sequences obtained may result from a contamination of sperm protein preparation by the epididymal fat pad originating adipsin. This was conceivable if we consider that the adipsin MW deduced from the mRNA sequence is of 27 kDa. A band corresponding to 26kDa of an electrophoretic pattern of protein extracted from a

large amount of epididymal fat cushion was excised and submitted to N-chlorosuccisinimide proteolysis (NCS). This digestion did not generate fragments on the SDS-PAGE electrophoretic pattern whereas the P26h NCS digest generated a 22.4 kDa fragment. Moreover, only the P26h and its NCS digested fragment were detected by a P26h antiserum used to probe a corresponding immunoblot. The NCS digested fragment of the P26h was sequenced and the inner sequence revealed also a high homology level with the Adipsin. The intact 26 kDa from the fat pad protein extract was submitted to the same procedure and no N-terminal sequence was obtained.

RT-PCRs were performed with oligonucleotides derived from the cDNA sequence of the adipsin. A 710 bp fragment was amplified from the hamster testis, cloned and sequenced. Using this fragment as a probe, a Northern blot analysis was performed to determine in which tissues P26h transcription occurs. Total RNA of several tissues were extracted and submitted to blot-hybridization analysis. The Northern blots showed that the total P26h messenger RNA has 1081 bp and that it was transcribed exclusively in the testis. To confirm the presence of intact RNA in all samples, the same blot was probed with random cyclophilin DNA, and an intense signal was obtained in all samples (Fig. 2). By opposition, Northern blot analysis of mRNA prepared from human tissues revealed an abundant transcript in the epididymal tissues (Fig. 7).

A cDNA library was constructed in Lambda gt11 from the hamster testicular mRNA and from the human epididymal tissues. 10^5 clones of the primary library (5×10^5 clones) were directly screened with the 710 bp cDNA probes. The first screening allowed the detection of 32 positive clones from which 11 were used for a second and third screening. The size of the inserts was

determined by PCR and the longest insert (clone 2), was introduced in pBluescript SK(+/-) phagemid and sequenced. The P26h cDNA of 1081 bp has a 732 bp open reading frame (orf) starting with a ATG codon at position 124 and a TAG stop codon at position 856, followed by a poly-adenylation signal, and a poly A tail (Figs. 3A-3C). The sequence is numbered from the 5' end of the cDNA clone. The translation of the proposed orf is shown below the nucleotide sequence and encodes a peptide of 244 amino acids terminating by an amber codon.

Results

When purified P26h was submitted to Edman degradation, 27 of the 29 amino acids generated were identified. A 17 kDa fragment obtained by NCS proteolysis of P26h allowed the identification of 15 of the 26 amino acids analyzed, whereas the fragment obtained following CNBr treatment allowed the identification of 8 of 9 additional amino acids. For a total of 40 amino acids identified by Edman degradation of P26h peptides, 37 showed homology with a mouse adipsin sequence (Fig. 1). This protein has been shown to be a differentiation growth factor of mouse adipoblasts.

The deduced amino acid sequence predicted a 26 kDa MW protein which is in agreement with the mw of the P26h as determined by SDS-PAGE. The N-terminal sequence of P26h and of its generated peptides determined by Edman degradation (Fig. 1) are also in agreement with the amino acid sequence deduced from the cDNA (Figs. 4A-4B). The P26h amino acid sequence was compared with adipsin and a carbonyl reductase, which showed a homology of 85% and 86% respectively. The deduced amino acid sequence of the human homologue (Figs. 8A-8B, lower sequence) predicts a 209 amino acid peptide sharing the SDR characteristics with the hamster P26h (Figs. 8A-8B, upper sequence).

Expression of P26h mRNA is detected in the testis using non-radioactive *in situ* hybridization. Digoxigenin-labeled anti-sense probe revealed the expression of the P26h mRNA in the adult hamster testicular seminiferous tubules. By opposition to the Northern Blot analysis, *in situ* hybridization revealed a weaker signal along the epididymis, principally in the *corpus* portion. Digoxigenin-labeled sense probe was used as a control for nonspecific hybridization. The *in situ* hybridization confirms that a P26h transcript is predominant in the testis and, at a lower level, in the epididymis. *In situ* hybridization has been performed with digoxigenin-labeled RNA probes system using an anti-digoxigenin antibody that allows amplification of the signal and provides a more sensitive mRNA detection than the traditional Northern blot analysis. A faint labeling is detectable all along the epididymis, a much stronger signal being associated with the corpus (Fig. 5). In many species, the *corpus* region is known to be a more active epididymal segment for protein synthesis and secretion. According to the Northern blot analysis (Fig. 2), P26h which is found at high concentration in the proximal region of the hamster epididymis probably originates from the testicular fluid as a secretion product of the seminiferous tubules, as suggested by the *in situ* localization of the transcript (Fig. 6). This protein may also be secreted by the corpus epididymidis. A dual testicular and epididymal origin has been described for other proteins interacting with the spermatozoa during epididymal maturation. Whether the testicular and the epididymal P26h are identical or exist in different isoforms, as described for clusterin, remains to be determined.

Using the TNT™ coupled reticulocyte lysate system, *in vitro* translation was performed with circular

plasmid including P26h cDNA. A 26 kDa signal with total translation products was detected on SDS-PAGE. (Fig. 6A3). Total translation products were then submitted to immunoprecipitation with anti-P26h antibody, which permitted the detection of a unique signal of 26 kDa on SDS-PAGE. (Fig. 6A2). Total translation products were further submitted to NCS proteolysis. The NCS proteclysis generated a 17 kDa fragment on SDS-PAGE in agreement with the deduced amino acids sequence and the previous NCS proteolysis of purified P26h.

Discussion

One of the best documented physiological functions acquired by the spermatozoa during epididymal maturation is their ability to efficiently interact with the egg's *zona pellucida*. The inventors have been interested by these sperm surface modifications, mainly the addition of new surface proteins or the post-translational modifications of preexisting sperm components that are necessary to produce a functional male gamete. P26h is abundant in the luminal fluid of the proximal region of the hamster epididymis, its concentration decreasing along the transit. Contemporarily, P26h accumulates on the spermatozoa during the epididymal maturation. P26h is exclusively located on the sperm surface covering the acrosomal cap of the mature spermatozoa, the subcellular domain involved in *zona pellucida* binding.

A single spot in two dimensional gel electrophoresis was obtained, this single protein being recognized by the anti-P26h on corresponding Western blot. These two preparations of purified P26h, as well as proteolytic fragments, have been N-terminal sequenced by Edman degradation. All the amino acid sequences obtained showed high homology with mouse adipsin (Fig. 1). Adipsin mRNA has been shown to be present in high

quantities in the mouse epididymal fat pad. Proteins from huge amounts of epididymal fat pad were extracted and proceeded in parallel with cauda epididymal spermatozoa. Protein bands of 26-27 kDa were excised from preparative SDS-PAGE of proteins extracted from fat pad adipocytes and from cauda epididymal spermatozoa. Intact 26-27 kDa bands and proteolytic fragments generated by NCS (N-Chlorosuccinimide) digest were Western blotted and probed with the anti-P26h serum. The 26-27 kDa fat pad protein was undetectable with the anti-P26h antiserum (Fig. 2). Furthermore, the 26-27 kDa fat pad band and the P26h sperm protein were proceeded in parallel for N-terminal sequencing by Edman degradation. No signal was detectable when the fat pad protein was proceeded. From these results we can conclude that the N-terminal sequences obtained did not result from a contamination of sperm preparation by the epididymal fat pad.

Northern blot analysis reveals a major P26h transcript in testicular tissues of the sexually mature hamster (Figs. 3A-3C). This mRNA is undetectable in the other tissues analyzed, including the fat pad and the epididymis (Figs. 3A-3C). This was unexpected since it was previously reported that an *in vitro* translational product encoded by mRNA of the proximal region of the epididymis can be immunoprecipitated by anti-P26h antibodies.

The P26h being transcribed principally in testicular tissues (Fig. 2), a testicular cDNA was screened to clone the P26h cDNA. The longest transcript obtained from the library was sequenced and revealed a cDNA of 1081 bp coding for a 244 amino acids protein. The P26h cDNA shows high sequence homology with adipsin, as expected from N-terminal amino acid sequences and with a carbonyl reductase known to be expressed in

pig lung (Figs. 4A-4B). The deduced amino acid sequence also shows a high homology of 87% with the adipsin and 80% with the carbonyl reductase. Considering that P26h is a sperm protein involved in gamete interactions, the biological function of these two proteins was puzzling. Adipsin has been described as a differentiation factor of adipoblast in adipocytes and its expression has been shown to be inhibited by activators of protein kinase C. The carbonyl reductase is a homotetramer that catalyzes the oxidation of secondary alcohols and aldehydes. This enzyme has been shown to be expressed specifically in the lung and mainly distributed in the mitochondria. Notwithstanding the high level of homology with adipsin and carbonyl reductase, P26h shows a complete different tissue distribution. The P26h protein and its encoding mRNA are not expressed in the lung nor in the adipocyte (Fig. 2; Bérubé and Sullivan, *supra*). Adipsin and carbonyl reductase are known to be members of the short-chain dehydrogenase/reductase (SDR) superfamily and P26h shows some of their properties.

The Short-Chain Dehydrogenase/Reductase superfamily (SDR) is formed by a variety of different proteins that exhibit residue identities of only 15-30%. This low level of sequence identity between the members indicates an early divergence. This is reflected by the wide range of functions fulfilled by the members of this superfamily. There are three classes of enzymes covering a wide range of EC numbers: 1, 4.2, 5.1, and 5.3, as well as members with unknown functions. Two consensus sequences are conserved in this family, the NAD(H) or NADP(H) binding domain, a N-terminal segment GlyXXXGLYXXGly, and the catalytic domain having the sequence TyrXXXLys. The P26h deduced amino acid sequence possesses these consensus domains as well as

the Gly 129, Ser 136 and Pro 179, which are conserved in more than 90% of the SDR family members (Figs. 4A-B).

Polyclonal antibodies have been produced against P26h and used to document the function of this sperm protein during the fertilization processes in the hamster. When added to an *in vitro* fertilization medium, the antibodies anti-P26h inhibit in a dose-dependent manner the sperm-*zona pellucida* interaction. Furthermore, active immunization of male hamsters against the purified P26h results in an immune response associated with reversible infertility. Using the anti-P26h antiserum, a human counterpart of P26h has also been identified and was showed to be absent from sperm of men presenting idiopathic infertility. In humans, this protein is also acquired by the spermatozoa during the epididymal transit. Taken together, these results clearly demonstrate the involvement of this sperm protein in the processes leading to fertilization. The P26h preparation that shows immunocontraceptive properties is the same than the one used to determine N-terminal sequence by Edman degradation (Fig. 1). Furthermore, the polyclonal antiserum that allowed us to document the function of P26h in the processes of fertilization also reacts with the translational product encoded by the sequenced cDNA (Fig. 6). This clearly demonstrates that this SDR member is involved in mammalian sperm-egg interaction.

The mammalian spermatozoon is a highly polarized cell characterized by well-defined membrane domains. Many sperm surface proteins have been proposed to play a role during the cascade of events occurring when the male gamete reaches the oocyte. Different sperm proteins have been proposed as candidates involved in *zona pellucida* binding. Some of them show enzymatic activity such as proacrosin, a trypsin like protease, a mannosi-

dase, a galactosyltransferase and P95, a hexokinase. The catalytic activity of these enzymes may not necessarily be involved in *zona pellucida* interaction; it is rather the substrate affinity that mediates this interaction. The biological function played by these proteins in gamete interactions is thus quite different from their enzymatic activity defined by their catalytic activity in cell metabolism. This discrepancy is reflected by their subcellular localization on the spermatozoon. To mediate *zona pellucida* recognition these enzymes must be localized at the sperm surface where they are classically known to be intracellular. This is well illustrated by the extracellular oriented sperm membrane mannosidase and galactosyltransferase, as well as by hexokinase, which is at the surface of the mouse spermatozoa where it is known to be associated with the mitochondrial membrane. Like these potential *zona pellucida* ligands, P26h is localized at the hamster sperm surface, to the membrane domain covering the acrosome.

P26h belongs to the SDR superfamily, which is characterized by highly different members with a low level of identity. This reflects distant duplications and early divergence. As a consequence, the SDR family represents a great diversity in enzymatic activities and functions. An interesting example of an alternative function for an enzyme is glyceraldehyde -3-phosphate dehydrogenase. This protein, which is classically known as a glycolytic enzyme, has been shown to act as a t-RNA binding protein with a function in cytoplasmic trafficking. SDR divergence is favorable for the arising of new functions, and involvement in gamete interactions may be one of these.

Considering that P26h has been previously shown to be involved in gamete interaction and to possess

immunocontraceptive properties, cloning of a homologous cDNA in human allowed the identification of a human sperm protein with immunocontraceptive properties (Figs. 8A-8B).

The blood-testis barrier is not present in the epididymis, allowing the neutralization of spermatozoa following immunization against an antigen involved in post-testicular maturation of the male gamete. The fact that the human sperm protein is specifically expressed in the epididymis (Fig. 7) strongly supports its potential as an immunocontraceptive target.

The present invention will be more readily understood by referring to the following example, which is given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Immunocontraception vaccine

The human counterpart of the hamster cDNA P26h encodes for an epididymal-specific protein that is important in sperm function. It is possible to target this protein by specific antibodies using an immunocontraceptive approach. Men may be immunized with a peptide corresponding to the epididymal protein. This peptide may be chosen with regards to its antigenic properties. An immune response against that specific peptide may occur and with no side effects since the selected peptide shows high specificity for a sperm-epididymal protein. The antibodies may reach the spermatozoa within the excurrent duct (epididymis) since the blood-testis barrier is not present at the level of the epididymis. The antibodies may neutralize the fertilizing ability of the spermatozoa as already shown with the hamster P26h and may confer an immunocontraceptive protection.

The peptide may be coupled to a carrier that may modulate the half-life of the circulating peptide. This may allow control on the period of contraceptive protection. The peptide-carrier may be emulsified in an adjuvant and administrated by an appropriate immunization route.

In men under such an immunocontraceptive regimen, the circulating titer of anti-peptide antibodies may be an indication of contraceptive efficiency. Reversibility may be predicted by standard immunological determination of the titer of antibodies specific to the specific peptide.